ANNOTATIONES ZOOLOGICAE JAPONENSES
Volume 53, No. 2—June 1980

Published by the Zoological Society of Japan

Taxonomic Studies on Two Heliozoans, *Echinosphaerium akamae* sp. nov. and *Echinosphaerium ikachiensis* sp. nov.

With 11 Text-figures

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ABSTRACT Two Japanese strains (MA and YI) belonging to the genus *Echinosphaerium* were found to be the new species in the present study. Comparing them with the already-published and related species, *E. nucleofilum* Barrett, 1958, the present two strains were studied on the sizes of cell body and endoplasm, ratios of diameter of endoplasm to thickness of ectoplasm, structure and function of axopodia, number and other features of nuclei, nucleo-cytoplasmic indices calculated from the volumes of either cell body or endoplasm, cell fusion reaction, and cell-substrate adhesion. Furthermore, it became one of the determinative factors that the present two strains did not result in fusion reaction between them and between either of them and *E. nucleofilum*. Such detailed examinations led us to make a conclusion that they should be new species and to name *E. akamae* and *E. ikachiensis*. A key to the species of the genus *Echinosphaerium* was proposed in the present study, because it had never been published before.

INTRODUCTION

The originally named *Actinosphaerium* belongs to the order Actinophryida, the subclass Heliozoia, the class Actinopodea, the superclass Sarcodina, and the subphylum Sarcomastigophora (Corliss, 1967; Honigberg *et al.*, 1964). With regard to the genus name, however, *Actinosphaerium* has been replaced by *Echinosphaerium* since Hovassé (1965) proposed. Therefore, the name *Echinosphaerium* is preferred to be employed in the present study.

Up to the present time four species belonging to the genus *Echinosphaerium* have been reported; *E. eichhorni* (Ehrenberg, 1840), *E. arachnoideum* (Penard, 1904), *E. portuum* (Kufferath, 1952), and *E. nucleofilum* (Barrett, 1958). In our earlier studies, on the other hand, the strain MA of *Echinosphaerium* has been employed

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to examine the dis- and re-assembly of axonemal microtubules (Shigenaka, 1976; Shigenaka, Tadokoro & Kaneda, 1975; Shigenaka, Watanabe & Kaneda, 1974; Toyohara, Shigenaka & Mohri, 1978), in addition to the cell functions such as cell division (Shigenaka & Toyohara, 1976; Suzaki, Shigenaka & Takeda, 1978), cell fusion (Shigenaka & Kaneda, 1979; Shigenaka, Maruoka & Toyohara, 1978; Shigenaka, Ogura & Maruoka, 1976; Toyohara, Maruoka & Shigenaka, 1977), and broom and windmill formation of axopodia (Toyohara, Shigenaka & Utsumi, 1977).

However, our further taxonomic and morphological studies on this strain and the other unpublished strain (YI) reached the conclusion that they might be the new species, because they differed clearly from each other and also from the already-known four species of *Echinosphaerium*. In consequence, the present paper aims to report the characteristics of these two new species and to propose the following names for them: *E. akamae* and *E. ikachiensis*. Furthermore, a key to the species of the genus *Echinosphaerium* is designed here because it has never been given before.

MATERIALS AND METHODS

Organisms. Living organisms of the two new species were originally collected and isolated from the following two locations: i) *E. akamae*: a ditch passing through the campus of Fukuoka University of Education, Akama, Munakata-cho, Fukuoka-ken; and ii) *E. ikachiensis*: a small pond located at the precincts of Himuro Shrine, Ikachi, Yanai-shi, Yamaguchi-ken, Japan. American strain of *E. nucleofilum* originally reported by Barrett (1958) was purchased directly from the Carolina Biological Supply Company, Elon College, Burlington, North Carolina 27215, U.S.A. For the subsequent description, abbreviations of MA (Munakata-Akama), YI (Yanai-Ikachi), and EC (Elon College) will be used here after their original geographic locations for *E. akamae*, *E. ikachiensis*, and *E. nucleofilum*, respectively.

All of these heliozoans were cultured at 20 ± 1°C, by employing 0.01% Knop solution containing 0.242 mM $Ca(NO_3)_2$, 0.141 mM KNO_3, 0.058 mM $MgSO_4$, and 0.105 mM KH_2PO_4. Small ciliates and flagellates (*Paramecium bursaria*, *P. aurelia*, *Tetrahymena thermophila*, *Chilomonas paramecium*, and so on) were given to the culture medium as food sources. Sub-culturing was carried out every one or two weeks.

Isolation and staining of nucleus. After each individual was placed onto a slide glass with a small amount of culture medium, it was treated with 1% Triton X-100, agitated slightly by a finger tip, stained with Azur C, and covered with a coverslip. Such preparations made possible to observe the nuclei without any loss and distortion and to visualize the nucleoli very clearly. Furthermore, it was found that all nuclei obtained from one individual appeared not to be different in shape, size and other features from one another.

Calculation of nucleo-cytoplasmic index. The nucleo-cytoplasmic index was

calculated as follows: The diameters of cell body and endoplasm were firstly measured at the living state of each organism followed by calculating the volumes of cell body (Vc) and endoplasm (Ve). Then, the already measured organism was fixed with the glutaraldehyde fixative recommended by Shigenaka, Roth and Pihlaja (1971) without the subsequent OsO_4 fixation, rinsed with distilled water, stained with Feulgen nuclear reaction (Shigenaka & Toyohara, 1976), dehydrated with graded ethanol series, and mounted with Biolite or balsam. Such preparations were found to allow to count the number of all nuclei included in each nucleus so that the volumes of all nuclei (Vn) might be calculated. Finally, the two kinds of nucleo-cytoplasmic index based on the volume of cell body (NPc) or endoplasm (NPe) were gained from the following well-known formulae; $NPc=Vn/(Vc-Vn)$ or $NPe=Vn/(Ve-Vn)$.

Calculation of fusion index. Forty organisms of each species were pipetted out and transferred into each well of the depression slides, which were maintained in the moist chamber at $20\pm1°C$. In the five similarly-prepared wells, the time-lapse changes of the number of organisms were examined every 2 hrs for the duration of 40 hrs. The same experiment was repeated three times for each species. From these data, the fusion index (FI) was calculated according to the following formula (Okada, 1962); $FI=N_0/N_t-1.0$ [N_0, original cell number; N_t, cell number at each time].

Electron microscopy. The organisms were fixed by the method of Shigenaka, Roth and Pihlaja (1971), dehydrated with a graded ethanol series, and embedded in the low-viscosity embedding medium (Spurr, 1969). Ultra-thin sections were prepared with glass knives by using a Porter-Blum ultramicrotome (MT-1), mounted on the Neoprene W-coated grids, and stained with 3% uranyl acetate in H_2O for 10 min and Reynolds' lead citrate stain (Reynolds, 1963) for 5 min. They were finally examined under an electron microscope (JEOL, JEM-100S) operating at 100 kV.

Results and Discussion

General features. The present two species are usually spherical in form but tend to become discoidal or hemi-spherical when they attach to the culture dish or other substrate as seen in other heliozoans (Barrett, 1958; Suzaki, Shigenaka & Takeda, 1978). They are also characterized by many radiating axopodia, in which hundreds of microtubules are contained as cytoskeletal elements and the cytoplasmic contents undergo streaming called saltatory movements (Roth, Pihlaja & Shigenaka, 1970; Shigenaka, Tadokoro & Kaneda, 1975; Shigenaka, Watanabe & Kaneda, 1974; Tilney & Porter, 1965). The cytoplasm of these species is differentiated into the coarsely vacuolated ectoplasm and the less transparent and granulated endoplasm. Moreover, a few to hundreds of nuclei are scattered just inside the periphery of endoplasm. From these general features, it is considered that

Table 1
Distinctive features in the four known species of the genus *Echinosphaerium*.

Species	Diameter of cell body(μm)	Endoplasmic differentiation	Nuclei	Pseudopodia	Habitation
E. eichhorni	200–300	Distinct	Scattered in the periphery of endoplasm; 12–20 μm ø	One kind (axopodia)	Fresh-water
E. nucleofilum	230–400	Distinct	Similar to that of *Actinophrys sol*; 4–8 μm ø	One kind (axopodia)	Fresh-water
E. arachnoideum	70– 80	Not dintinct	Smaller in number	Two kinds	Fresh-water
E. portuum	30	Distinct	Unknown	One kind (axopodia)	Sea-water

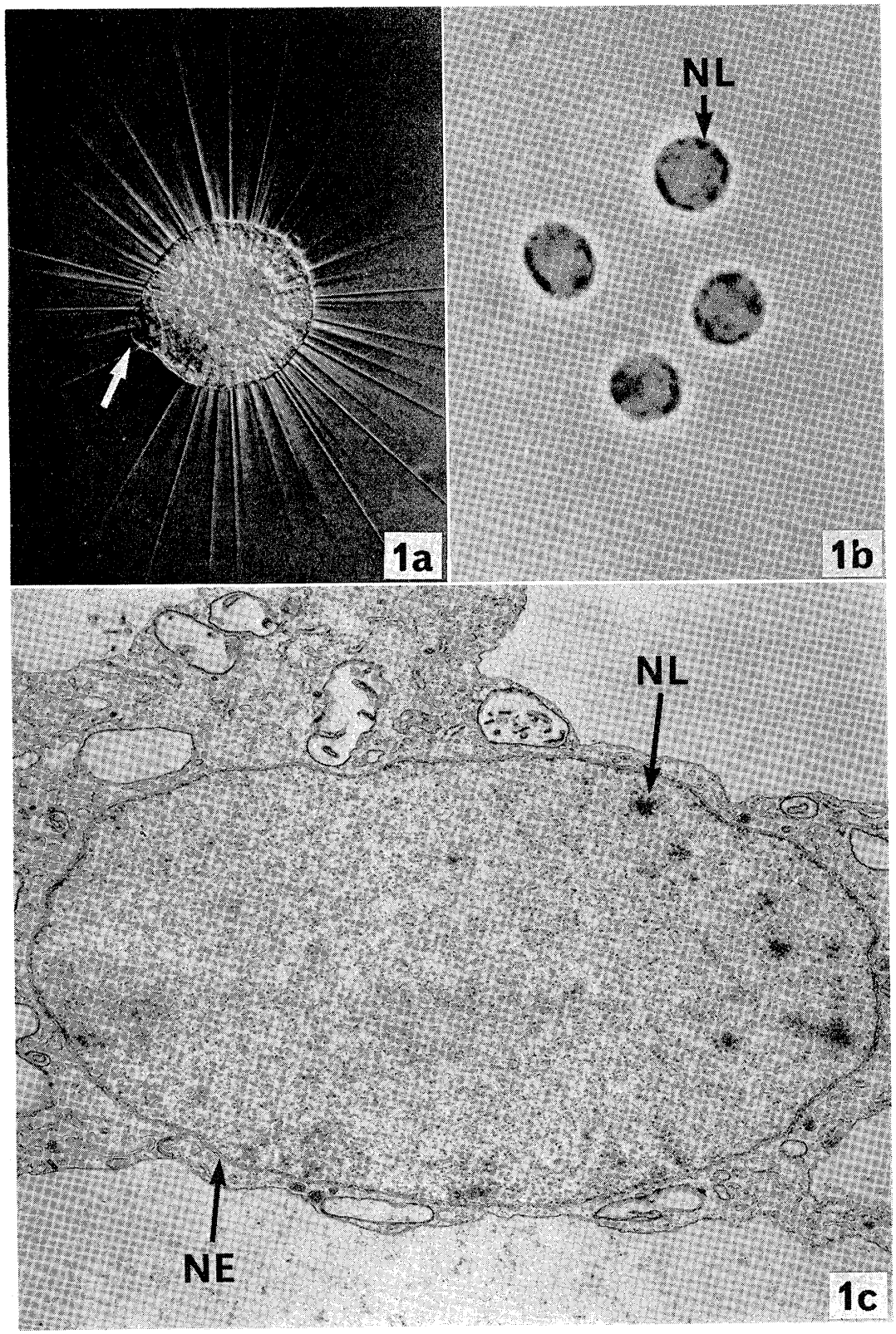
the present two species must belong to the genus *Echinosphaerium* (Hovassé, 1965).

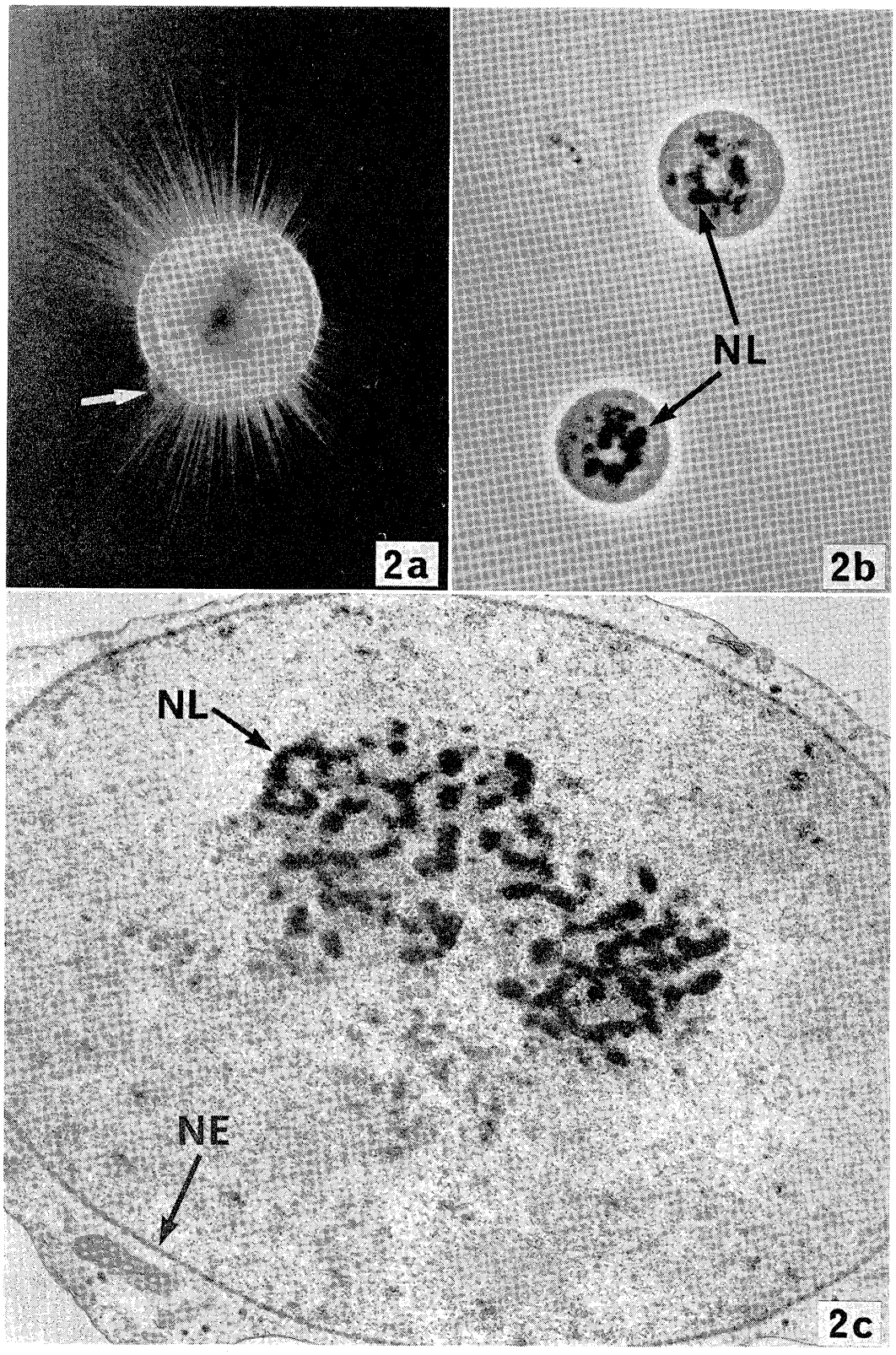
Although it has been reported that the genus *Echinosphaerium* inhabits either fresh-water (Barrett, 1958) or sea-water (Kufferath, 1952) as shown in Table 1, the present two species were found to inhabit fresh-water ponds and ditches just as in *E. eichhorni*, *E. nucleofilum* and *E. arachnoideum*. It should be noted that among these fresh-water species, only *E. arachnoideum* appears to be quite different from the other two and the additional present species; the former does not differentiate clearly into two parts in its cytoplasm, and furthermore, bears two kinds of pseudopodia, one straight and very long and the other filiform and anastomosing (Penard, 1904; Kudo, 1966). In consequence, the present types of organisms seem to be closely related to *E. eichhorni* and *E. nucleofilum*. Morphological and other typical features of the two new species will be hereafter described particularly in comparison with *E. nucleofilum* called strain EC here.

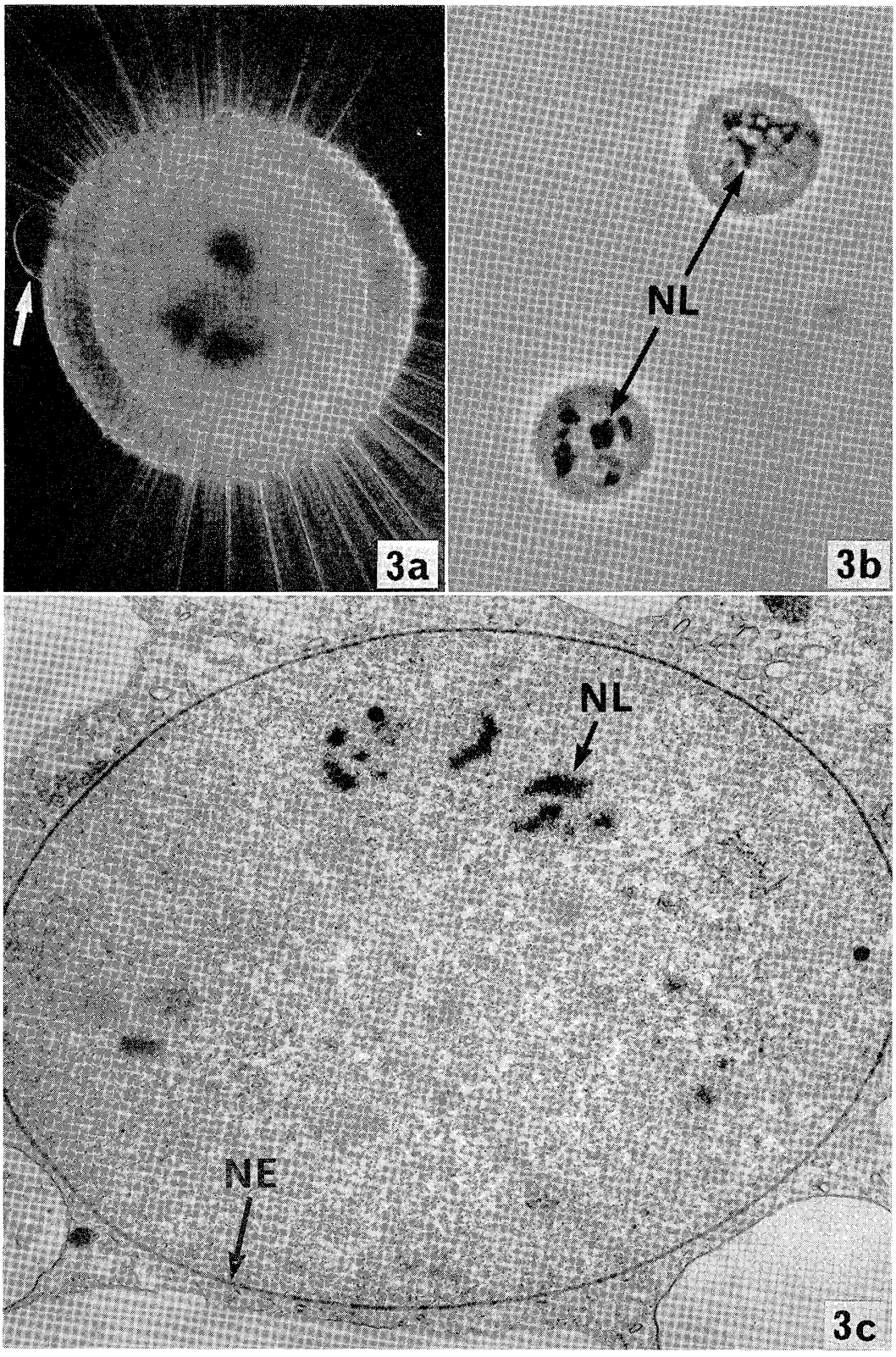
Fig. 1 (on p. 107). Light and electron micrographs of *Echinosphaerium nucleofilum* Barrett, 1958. —— 1a: A living cell, showing a typical contractile vacuole with an arrow (×135). 1b: Isolated and Azur C-stained nuclei, showing the nucleoli (NL) located just inside the nuclear envelope (×1,270). 1c: A cross section through the nucleus, showing the nuclear envelope (NE) with wavy appearance and nucleoli as electron-dense regions (×13,400).

Fig. 2 (on p. 108). Light and electron micrographs of *Echinosphaerium akamae* sp. nov. —— 2a: A living cell, showing a contractile vacuole (arrow) (×135). 2b: Isolated and Azur C-stained nuclei, showing the nucleoli (NL) inside the nucleus (×1,270). 2c: A cross-sectioned nucleus, showing the smooth nuclear envelope (NE) and nucleoli as big masses (×11,200).

Fig. 3 (on p. 109). Light and electron micrographs of *Echinosphaerium ikachiensis* sp. nov. —— 3a: A living cell, showing a contractile vacuole (arrow) and food materials (*Paramecium bursaria*) as three dark fields (×135). 3b: Isolated and Azur C-stained nuclei, showing the nucleoli (NL) inside the nucleus (×1,270). 3c: A cross section through the nucleus, showing the smooth nuclear envelope and nucleoli as electron-dense materials (×8,900).







Sizes of cell body or endoplasm. Light micrographs of the living organisms derived from the three species, *E. nucleofilum* (EC), *E. akamae* (MA), and *E. ikachiensis* (YI), are depicted in Figs. 1–3, showing the above-mentioned morphological

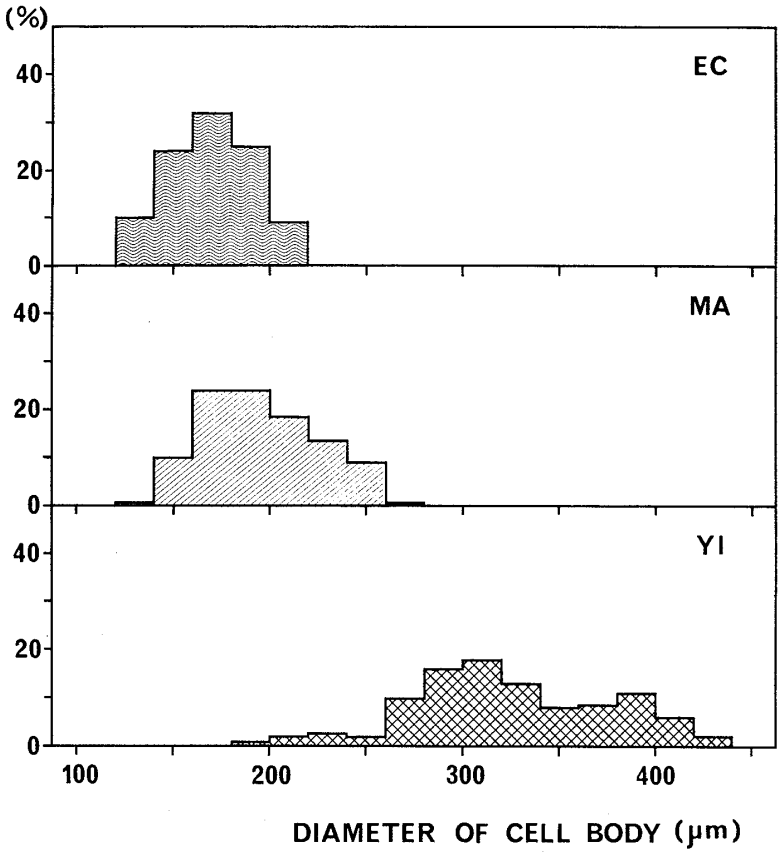


Fig. 4. Comparison of the diameters of cell body among the three species, *E. nucleofilum* (EC), *E. akamae* (MA), and *E. ikachiensis* (YI). Abscissa: diameter of cell body (μm); ordinate: frequency (%).

Table 2
Comparison of two new species with the most closely related species, *E. nucleofilum*.

Species	Diameter of cell body (μm)	Diameter of endoplasm (a) (μm)	Thickness of ectoplasm (b) (μm)	Ratio of (a) to (b)	Ratio of axopodial length to diameter of cell body
E. nucleofilum	123–210 (172)	56–147 (95)	31– 92 (39)	0.6–4.7 (2.6)	1.3–2.3 (1.7)
E. akamae sp. nov.	138–273 (195)	82–244 (144)	18– 44 (26)	1.9–14.0 (6.0)	0.5–1.1 (0.8)
E. ikachiensis sp. nov.	186–436 (335)	125–343 (246)	22– 57 (45)	3.3–15.7 (5.8)	0.3–0.8 (0.5)

Average values are given in parentheses.

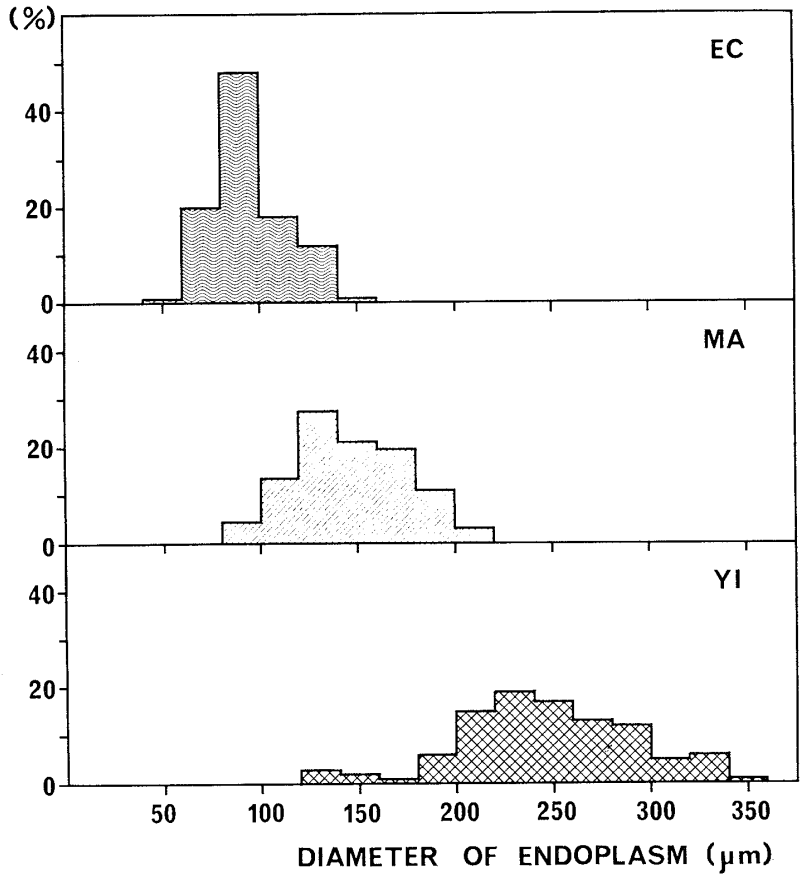


Fig. 5. Comparison of the diameters of endoplasm among the three species (EC, MA and YI). Abscissa: diameter of endoplasm (μm); ordinate: frequency (%).

features and actively-functioning contractile vacuoles. Cell sizes of living organisms were measured by sampling from 3 to 5-day old cultures, because they tend to become larger with repeatedly occurred fusion reactions about one week after inoculation. As shown in Fig. 4 and Table 2, the diameters of cell body in EC, MA and YI were found to vary from 123 to 210 μm (172 μm in average), 138 to 273 μm (195 μm), and 186 to 436 μm (335 μm), respectively. From these data, it may be considered that the average volume of cell body is in the order of EC$<$MA$<$YI, and that the variation of cell sizes in MA is quite similar to that in EC but rather smaller than in YI.

As shown in Fig. 5 and Table 2, the endoplasmic diameters in EC, MA and YI were found to vary from 56 to 147 μm (95 μm in average), 82 to 244 μm (144 μm), and 125 to 343 μm (246 μm), respectively. This means that the endoplasmic diameter is also in the order of EC$<$MA$<$YI just as in the cell volume described above. Particularly in MA, the sizes of cell body are very close to those in EC but the endoplasmic sizes are usually much larger than in EC and demonstrate the intermediate values between other two species, EC and YI (compare Fig. 4

with Fig. 5). In consequence, inter-specific difference of the cell sizes had better be discussed from the two viewpoints of cell body and endoplasm, especially with respect to the ratios of diameter of endoplasm to thickness of ectoplasm as suggested by Barrett (1958). Such data are shown also in Table 2, revealing that the ratios in EC are the smallest among the three species, although it is still doubtful whether these ratios are the most useful to distinguish these species from one another.

Axopodial length and function. Only from the morphological viewpoints, it is quite difficult to distinguish the three species (EC, MA and YI) from one another; they are always radiating as the pointed needle-shaped extensions from the cell surface, containing mitochondria, electron-dense granules, and axonemal microtubules. However, Barrett (1958) has stressed that when the new species is determined, it is necessarily required to examine the ratios of axopodial length to diameter of cell body. Therefore, we examined these values for the present three species as shown in Table 2; MA and YI appeared to be within about the same range but different from those in EC.

In the present study, the axopodia were examined with respect to their function.

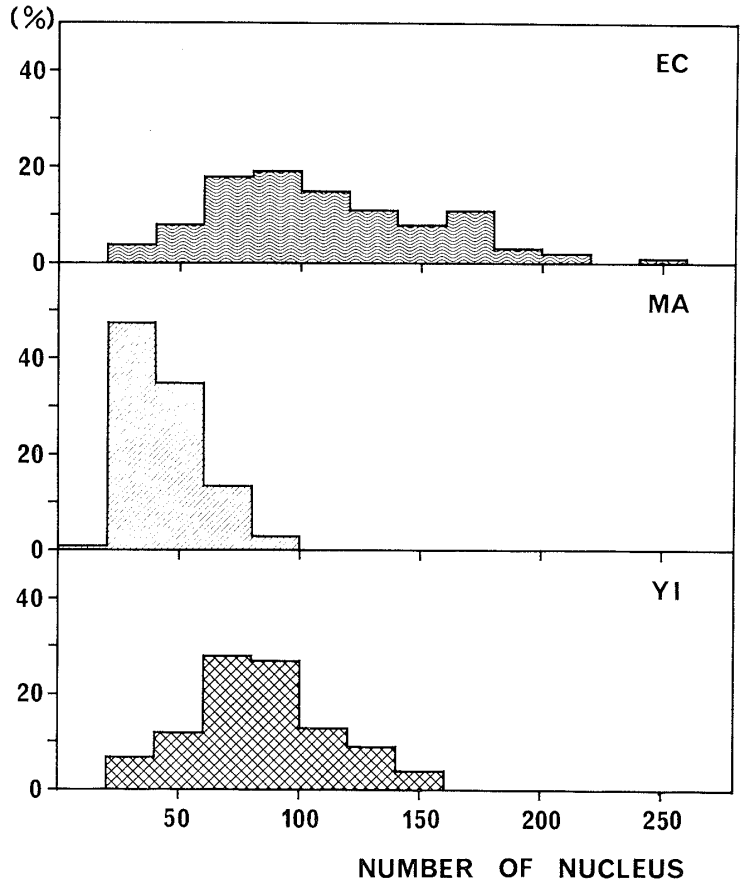


Fig. 6. Comparison of the numbers of nucleus among the three species (EC, MA and YI). Abscissa: number of nucleus; ordinate: frequency (%).

As the results, they appeared to be different from one another, especially in their functions such as cell movement, cell fusion, and cell-substrate adhesion as described below. Moreover, these species were determined to be distinguishable from the results that the inter-species cell fusion never took place even if they were enforced to touch by a slight centrifugational force.

Number and morphological feature of nucleus. At first, the numbers of nuclei were examined in three species, the results being demonstrated in Fig. 6. Only from this point, it seemed to be difficult to distinguish them. When compared with one another, however, their variations appeared to become larger in the order of MA, YI and EC; as far as the variations were examined, they were 6 to 98 in MA, 22 to 155 in YI, and 21 to 257 in EC.

On the other hand, the morphological features of nucleus were found to be distinguishable from one another as shown in Figs. 1–3. That is to say, the isolated nuclei in EC (Fig. 1b) were quite different from those in MA (Fig. 2b) and YI (Fig. 3b), especially in the outlines of nuclear periphery and the structures and locations of Azur C-positive nucleolus; in the EC, the nuclear envelopes usually appeared with the irregular contours and the underlaid granular nucleoli. With respect to these points, the MA appeared to be quite similar to YI; the nuclear envelopes were featured with smooth contours and the nucleoli did not attach to the envelopes, demonstrating the granular or fibrous structures decorated with fuzzy materials. These results were again surveyed by means of an electron microscope; the EC was characterized by the nuclear envelope with wavy appearance

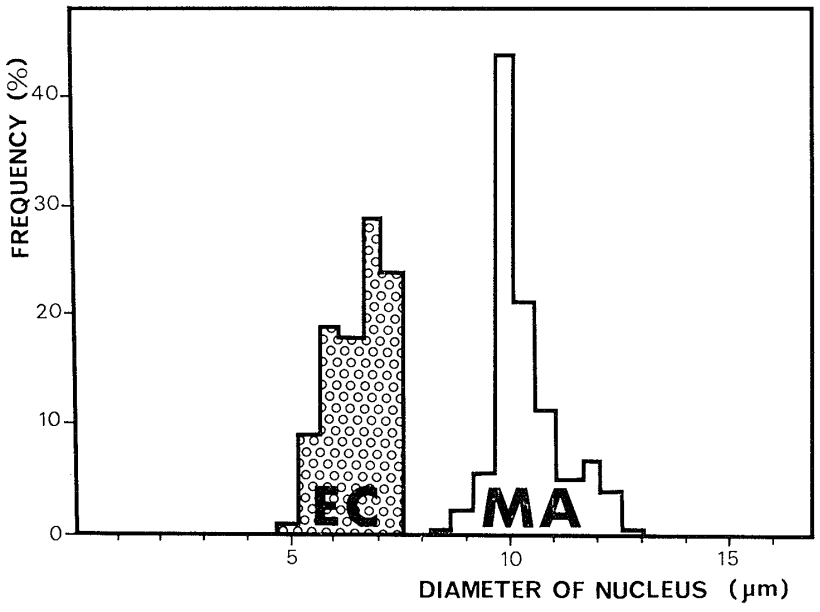


Fig. 7. Comparison of the diameters of nucleus between the two species (EC and MA). Abscissa: diameter of nucleus (μm); ordinate: frequency (%).

and the nucleoli located very close to the envelope (compare Fig. 1c with Figs. 2c and 3c). As far as the structures of nucleoli were concerned, these three species were not distinguishable from one another.

Finally, the diameters of nuclei were examined at the isolated condition. The results are demonstrated in Fig. 7, revealing that the nuclei of EC were smaller in diameter than those of MA. Although the data are not shown here, the nuclei of YI were about the same in diameters as in MA.

Nucleo-cytoplasmic index. Among the three species, the so-called nucleo-cytoplasmic indices were compared with one another on the basis of two kinds of volumes (cell body or endoplasm), because the ectoplasmic region in *Echinosphaerium* was occupied by a number of large vacuoles. The results are shown in Figs. 8–9. When based on the volume of cell body, the indices (NPc) in EC appeared to be approximately within the same range as in YI, MA being slightly different from EC and YI (Fig. 8). On the other hand, the results calculated from the volume of endoplasm revealed that the indices (NPe) in MA were about the same as in YI but rather different from those in EC (Fig. 9).

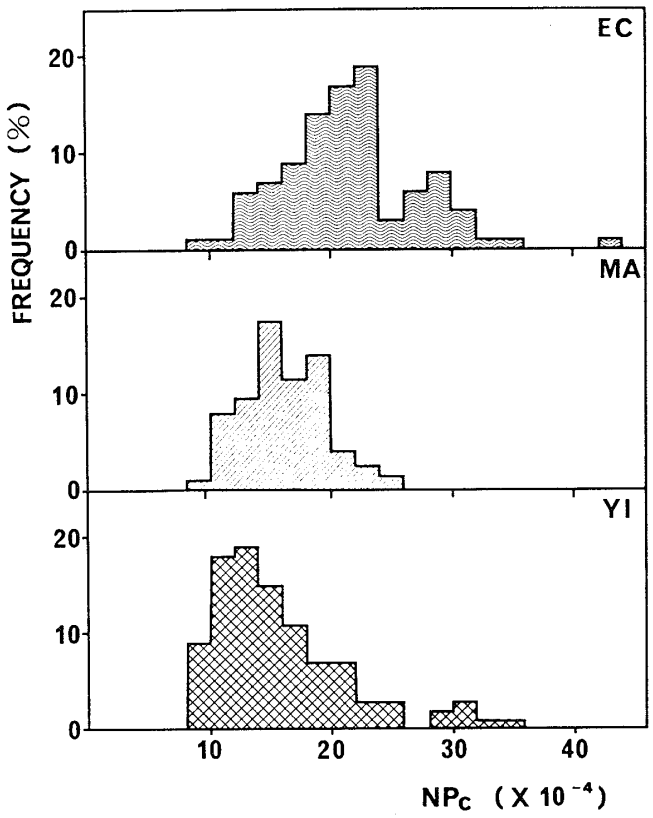


Fig. 8. Comparison of the nucleo-cytoplasmic indices (NPc) calculated from the volumes of cell body among the three species (EC, MA and YI). Abscissa: nucleo-cytoplasmic index ($\times 10^{-4}$); ordinate: frequency (%).

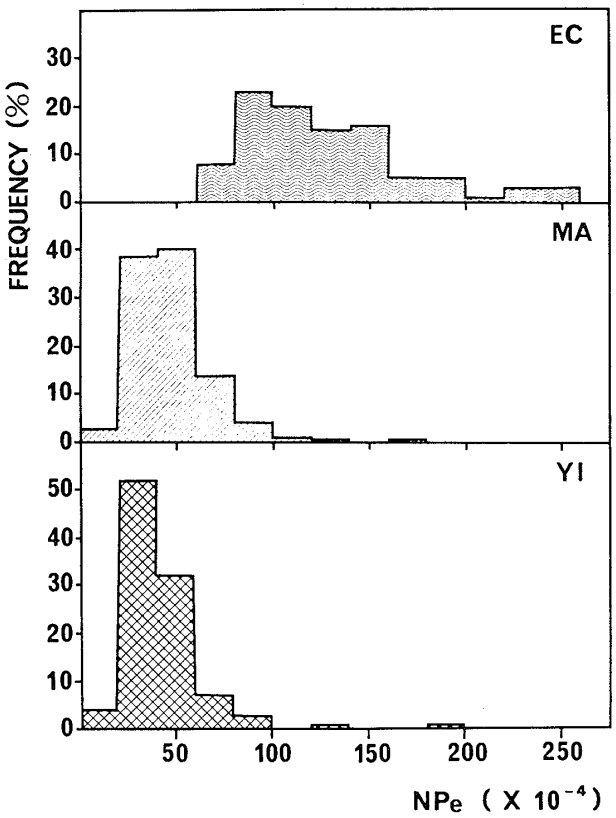


Fig. 9. Comparison of the nucleo-cytoplasmic indices (NPe) calculated from the volumes of endoplasm among the three species (EC, MA and YI). Abscissa: nucleo-cytoplasmic index ($\times 10^{-4}$); ordinate: frequency (%).

Considering the substantial and cell biological standpoints, the latter NPe appears to be much more meaningful for heliozoans themselves than the former, NPc, because the ectoplasmic matrix is negligibly small when compared with the endoplasm (refer to the light-micrographs in Figs. 1–3 and the ratio of the diameter of endoplasm to the thickness of ectoplasm in Table 2).

Cell fusion reaction. The time-lapse changes of fusion indices were examined among the three species. As shown in Fig. 10, the cell fusion activities appeared to be the highest in MA but about the same in EC and YI. Therefore, MA is supposed to be the most useful species in order to survey the cell fusion mechanism; we have much studied on the fusion reaction in this species (Shigenaka & Kaneda, 1979; Shigenaka, Maruoka & Toyohara, 1978; Shigenaka, Ogura & Maruoka, 1976; Toyohara, Maruoka & Shigenaka, 1977). On the other hand, EC has also been studied on the fusion reaction, revealing that the cold treatment, in the presence of Mg^{2+}, is necessarily required for inducing the fusion reaction especially in this species (Vollet & Roth, 1974; Vollet, Roth & Davidson, 1971, 1972). However, such a pre-treatment is not required for inducing the fusion reaction in MA, divalent cations being capable of enhancing the reaction though (Shigenaka, Maruoka &

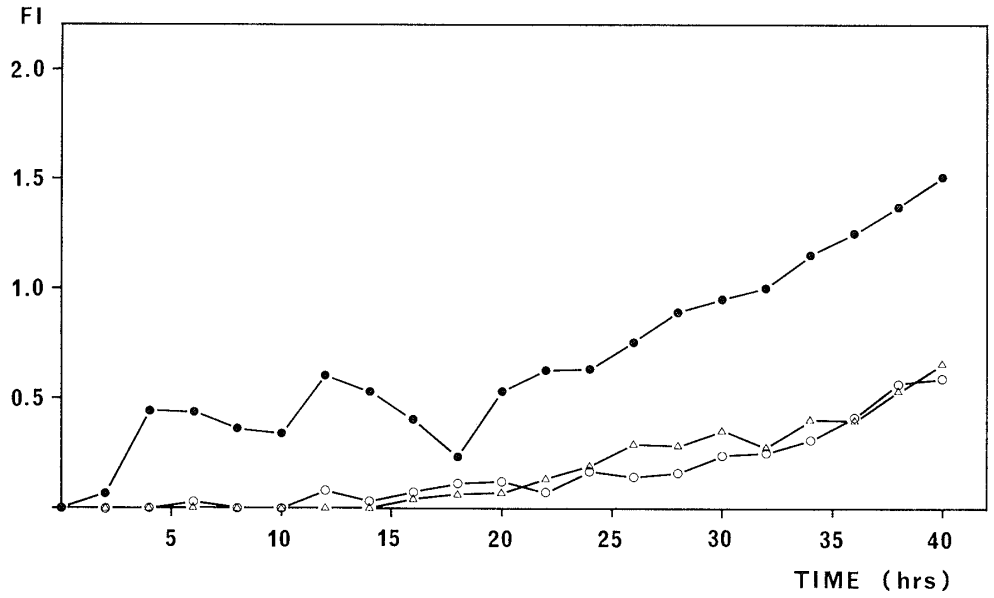


Fig. 10. Time-lapse changes of the fusion reaction in the three species, *E. nucleofilum* (○), *E. akamae* (●), and *E. ikachiensis* (△). Abscissa: time (hrs); ordinate: fusion index.

Toyohara, 1978).

Cell-substrate adhesion. As observed already in EC (Barrett, 1958), the present two species were also found to tend to adhere to the culture dishes. This type of reaction is generally termed as the plating reaction. Barrett (1958) has described that almost without exception large cultures in EC show 95 to 100% of the animals

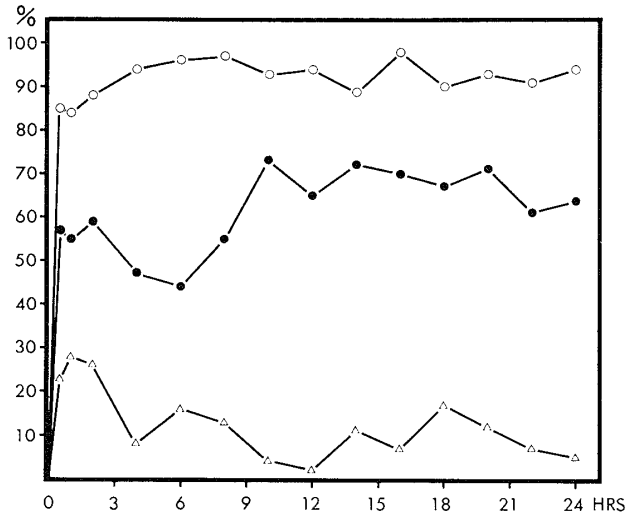


Fig. 11. Time-lapse changes of the cell-substrate adhesion in the three species, *E. nucleofilum* (○), *E. akamae* (●), and *E. ikachiensis* (△). Abscissa: time (hrs); ordinate: adhesion ratio (%).

attached, while in similar culture of *E. eichhorni* this is only rarely the case. Such a reaction of the present two species (MA and YI) was examined in time-lapse, revealing that it was quite useful for distinguishing them from one another, and that it was less conspicuous than in EC and varied even between MA and YI (Fig. 11).

Moreover, it was found by a simple pipetting procedure that the adhesive force to the substrate appeared to vary among these three species and seemed to become stronger proportionally to the frequency of cell-substrate adhesiveness and to be in the order of YI<MA<EC. This was actually demonstrated when the heliozoans were sub-cultured or experimented by pipetting. That is to say, the EC was easily torn away because it was attached more firmly to the substrate than in MA and YI.

Key to the Genus *Echinosphaerium*

1. Living in fresh-water 2
— Living in sea-water 8
2. Endoplasmic differentiation distinct 3
— Endoplasmic differentiation not distinct 7
3. Ratio of the diameter of endoplasm to the thickness of ectoplasm about 0.6–5: 1; 120–210 μm (originally reported as 230–400 μm) in diameter of the cell body; one kind of pseudopodia, axopodia; ratio of axopodial length to diameter of cell body, 1.3–2.3; nuclei similar to that of *Actinophrys sol* and 4–8 μm in diameter ..... *E. nucleofilum*
— Above-mentioned ratio, 2 or more: 1 4
4. Ratio of the diameter of endoplasm to the thickness of ectoplasm about 2–4: 1, 200–300 μm but sometimes up to 1 mm in diameter of the cell body; one kind of pseudopodia, axopodia; nuclei 12–20 μm in diameter ..... *E. eichhorni*
— Above-mentioned ratio 2–16: 1 5
5. Ratio of the diameter of endoplasm to the thickness of ectoplasm about 2–14: 1; 140–270 μm in diameter of the cell body; one kind of pseudopodia, axopodia; ratio of axopodial length to diameter of cell body 0.5–1.1; nuclei 8–13 μm in diameter ..... *E. akamae* sp. nov.
— Above-mentioned ratio 3–16: 1 6
6. 190–440 μm in diameter of the cell body; one kind of pseudopodia, axopodia; ratio of axopodial length to diameter of cell body 0.3–0.8; nuclei 9–15 μm in diameter ..... *E. ikachiensis* sp. nov.
7. 70 to 80 μm in diameter of the cell body; two kinds of pseudopodia, axopodia; one straight and very long and the other filiform and anastomosing; nuclei, smaller in number ..... *E. arachnoideum*
8. 30 μm in diameter of the cell body; one kind of pseudopodia, axopodia; endoplasmic differentiation, distinct ..... *E. portuum*

ACKNOWLEDGEMENTS

The authors wish to thank Misses A. Ashikari and M. Soda for their valuable discussion and technical assistance throughout the present study.

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